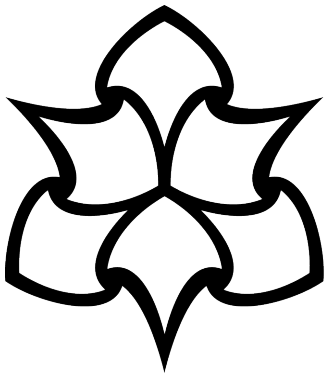

Exercise conditioning in old mice improves skeletal muscle regeneration
The following manuscript is in preparation for submission to the FASEB journal.
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Short title: Exercise improves regeneration

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Abstract

Skeletal muscle possesses the ability to regenerate following injury but is impaired or delayed with aging. Regardless of age, muscle retains the ability to positively respond to stimuli like exercise. We examined whether exercise is able to improve the regenerative response in skeletal muscle of aged mice. Twenty two month old male C57Bl/6J mice (n=20) underwent an 8wk progressive exercise training protocol (O-Ex) and were compared to old sedentary (O-Sed) and young sedentary (Y-Ctl) mice. Mice received injections of cardiotoxin into their tibialis anterior muscle. The TA were harvested prior to (O-Ex/O-Sed/Y CTL n=6), 10 days (O-Ex/O-Sed/Y D10 n=8) and 28 days (O-Ex/O-Sed/Y D28 n=6) post-injection. The average fibre cross sectional area (CSA) was reduced in all groups at D10 (CTL: O-Ex: 2499±140, O-Sed:2320±165, Y:2474±269; D10: O-Ex:1191±100, O-Sed:1125±99, Y:1481±167μm2; all $p<0.05$) but was restored to control values in O-Ex and Y-Ctl groups at D28 (O-Ex:2257±181, Y:2398±171 μm2, both $p>0.05$). Satellite cell content was greater at CTL in O-Ex (2.6 ± 0.4 SC/100 fibres) compared to O-Sed (1.0 ± 0.1 SC/100 fibres) ($p<0.05$). Exercise conditioning appears to improve the skeletal muscle's ability to regenerate following injury in aged mice.

Key words: skeletal muscle, regeneration, exercise, aging

Introduction

The loss of muscle mass and strength associated with advancing age (sarcopenia) can ultimately become debilitating (1). Developing interventions that target the mechanisms of age-related impairments in skeletal muscle growth/adaptation/repair is essential in improving the quality of life of the elderly. Satellite cells (SC) are resident skeletal muscle stem cells and play an important role in early postnatal muscle growth, formation of new fibers and maintenance of muscle mass (2). Historically, it was postulated that SC were necessary for skeletal muscle hypertrophy (3) however, more recent work highlights their redundancy in mediating this process in rodents (4). Although the role of SC in muscle remodelling remains debatable, they are essential in skeletal muscle regeneration (4,5). Depletion of SC in rodents leaves them able to hypertrophy to the same extent as wild type animals but their ability to regenerate skeletal muscle following injury is severely impaired (4). This points to a fundamental difference in SC with aging relevant to their roles in hypertrophy and regeneration following damage.

The regenerative process of skeletal muscle following injury is highlighted by several overlapping phases: the destructive/inflammatory phase, the repair phase and the remodeling phase (6). During the first phase of regeneration, the destructive phase, there is a degeneration of damaged fibres along with an influx of inflammatory cells (7). The influx of inflammatory cells not only leads to a clearance of muscle debris but also has a chemotactic role in SC migration to the site of injury (7). During the second or 'regenerative' phase, SC fuse to injured muscle fibres contributing to their repair, or fuse to each other to form new fibers (6). In the final phase, newly formed fibers or repaired fibers grow and ultimately re-establish contractility (6). In mice, regeneration of the tibialis anterior (TA) following injury, to control levels, has been consistently reported after ~21 days in young mice (8,9). However, existing evidence on whether old mice have the ability to fully regenerate their skeletal muscle following a similar injury is equivocal. Complete regeneration of the muscle following injury in old mice has been observed (8,10,11) while others report incomplete or delayed regeneration (12–14). More specifically, some studies demonstrate that although early regeneration is impaired in old mice, regeneration occurs to the same extent in both young and old when examined at later time points (8,10,11). These studies underpin the notion that delays in the inflammatory response and angiogenesis account for the early impaired regenerative response observed in old mice (10,11).

Although the extent to which aged skeletal muscle is able to regenerate following injury is debatable although it is widely accepted that SC content and function decreases in aged rodents. The decrease in SC content is thought to, at least in part, play a role in skeletal muscle atrophy associated with aging (15) and the impaired/delayed regeneration in aged skeletal muscle (8,16–19). Age-related contraction of the SC pool likely contributes to impaired regeneration, but there is mounting evidence suggesting that the systemic environment of old mice also plays a role in the loss of SC function (13,14,20). Models of parabiosis have elegantly demonstrated that when old skeletal muscles are exposed to a young systemic circulation, regeneration and SC proliferation is restored (14,20) and the deposition of fibrotic tissue is reduced (14). These data highlight the importance of extrinsic cues from the systemic environment in mediating improvements in skeletal muscle regeneration.

Several studies have explored the adaptive potential of aged mice to exercise stimuli. Following endurance exercise training in old mice there are improvements in spontaneous locomotion (17) and an increased life-span (21). In addition to improvements in more functional outcomes, an increase in SC content has consistently been reported following endurance training in young (22,23) and old rodents (17,19). Moreover, resistance training impedes the progressive loss of muscle associated with aging in rats (24) and prevents the age associated shift in fibre type distribution (25).

As SC are essential in muscle regeneration and endurance training results in an increase in SC content it stands to reason that exercise may result in improved skeletal muscle regeneration in old mice. Additionally, as described above, exposing old mice to a young systemic environment resulted in improved muscle regeneration. In a human model, exercise is also known to have pronounced systemic effects even in non-contracting muscles (26) and may promote a more youthful systemic environment contributing to improved muscle regeneration.

As described above, aged rodents maintain the ability to positively respond to exercise (17,19,21). Therefore the purpose of the current study was to determine the impact of endurance exercise training on skeletal muscle regeneration in old mice. The study design also enabled us to describe the effects of endurance exercise on the skeletal muscle of old mice and describe the effect of aging on skeletal muscle regeneration.

Materials and methods

Animals

Twenty-two month old adult male C57Bl/6J mice (n=10 Charles River, USA; n=10 Jackson Laboratories, Bar Harbor, ME, USA), were subjected to the 8 week exercise training protocol (O-Ex; n=20), mice were sacrificed at 24 months of age. Twenty-four month old adult male C57Bl/6J mice (Charles River, USA n=10; Jackson Laboratories, Bar Harbor, ME, USA n=10), were used as a sedentary group (O-Sed; n=20) and did not take part in exercise training. 8 wk old young adult male C57Bl/6J mice (Charles River, USA n=10; Jackson Laboratories, Bar Harbor, ME, USA n=10) were used as a young control group (Y-Ctl, n=20). All old mice were housed together in cages with no more than 4 animals; O-Sed and O-Ex animals were not housed separately. Old mice were housed in HEPA filtered clean cages. All young animals were housed together with no more than 5 animals per cage. Mice were provided with food and water *ad libitum*. Mice were kept on a 12-h light-dark cycle. Ethics approval was granted by the McMaster University Animal research Ethics Board and conformed to the standards established by the Canadian Council on Animal Care.

Exercise training protocol

Old mice were exercised trained (n=20) on an Exer 6M treadmill (Columbus Instruments, Columbus, OH, USA) 40 minutes/session, 3days/week for 8 weeks. Every exercise session was preceded by a 10 minute warm-up at 6 meters/minute

followed by a 5 minute cool-down at 6 meters/minute. Exercise training was progressive and began at 8.5 meters/minute (week 1) and increased to 15 meters/minute (week 8). Mice were encouraged to run on the treadmill with light electric shock and hindlimb stimulation when they stopped running.

CTX injection

To determine the effect of aging and exercise on aged skeletal muscle, animals from each group Y-Ctl, O-Ex and O-Sed were subjected to bilateral injections of 50 µl (10µM) of cardiotoxin (CTX) (Latoxan, Valence, France) into their TA muscle.

Animal sacrifice

Mice were briefly anesthetized with isoflurane (Abraxis Bioscience, Summit, NJ, USA), then euthanized via cervical dislocation. Both TA muscles were removed, one was frozen immediately in liquid nitrogen for RNA analysis while the other was mounted in Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) then frozen in liquid nitrogen pre-cooled isopentane for histology experiments. For baseline comparisons (CTL) mice not subjected to CTX injections were euthanized from each group, Y-Ctl, O-Ex and O-Sed (n=6, per group). Mice from the O-Ex group were euthanized 3 days following their last bout of exercise to minimize the possibility of observing acute exercise effects. To observe skeletal muscle regeneration, mice from each group were euthanized 10 (D10) (n=8, per group) and 28 days (D28) (n=6, per group) following CTX injection.

RNA isolation, reverse transcription and quantitative RT-PCR reaction

Total RNA was isolated from TA muscles using a combination of TRIzol (Invitrogen) and E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA).

Whole TA muscles were homogenized using 1 ml of TRIzol reagent in Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA),with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) twice for 40 s at 6 m/s. Samples were stored at -80□C until further processing. Samples were thawed and 200 µl of chloroform was added to each sample and mixed vigorously for 15 s, incubated for 5 min at room temperatures and then centrifuged at 12 000g for 10 min at 4°C. The upper aqueous phase was removed and RNA was isolated following the E.Z.N.A. Total RNA Kit 1 manufacturer’s instructions. RNA was reverse transcribed using a commercially available kit (high-capacity cDNA reverse transcription kit; Applied Biosystems, Carlsbad, CA, USA) following manufacturer's instructions using an Eppendorf Mastercycler ep gradient thermal cycler (Eppendorf, Mississauga, ON, Canada).

Quantitative RT-PCR reactions were prepared using the epMotion 5075 Eppendorf automated pipetting system (Eppendorf) and conducted in duplicates in an Eppendorf realplex2 Master Cycler ep gradient S (Eppendorf). All samples were normalized to RPS11 fold changes in gene expression were calculated using the ΔΔCt method and expressed in relation to Y-Ctl, CTL values. The primer sequences are as follows RPS11: forward 5'-*CGTGACGAAGATGAAGATGC*-3', reverse 5'-*GCACATTGAATCGCACAGTC*-3'; VEGF: forward 5'-*TTACTGCTGTACCTCCACCA*-3', reverse 5'-*ACAGGACGGCTTGAAGATGTA*-3'.

Immunohistochemistry

7μm thick TA muscle cross sections were prepared from OCT embedded muscle. To determine SC content, fibre cross sectional area (CSA) and proportion of regenerating fibres identified as fibres with centrally located nuclei, TA muscle cross sections were stained using the Vector Laboratories (Burlington, ON, Canada) mouse-on-mouse immunodetection kit as per the manufacturer's instructions, with minor modifications (overnight blocking step). Slides were treated with primary antibodies for Pax7 [neat; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA] and laminin (1:1000; ab11575 Abcam). Pax7 was detected using streptavidin-594 fluorochrome, 1:500; Invitrogen, Molecular Probes and laminin was detected using Alexa Fluor 647goat anti rabbit (1:500). Nuclei were visualized with DAPI. Images were taken at 20x with a CoolSNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY, USA). Images were analyzed using Nikon NIS elements AR software. CSA and the proportion of regenerating fibres were determined on an average of >200 fibres/animal, SC content was determined on an average of >700 fibres/animal.

To determine capillarization, TA muscle sections were stained with CD31 and laminin. Slides were treated with PFA for 10 min, washed in PBST then incubated in block (1% BSA and 10% goat serum) for 90 min. Slides were incubated in CD31 primary antibody (Abcam 28364) overnight at 4ºC. Slides were washed and CD31 was detected using Alexa Fluor 488 goat anti rabbit (1:500), slides were re-fixed in 4% PFA washed and incubated with laminin primary antibody (1:1000; ab11575 Abcam) for 2 h at room temperature. Nuclei were detected using DAPI. The capillary-to-fiber ratio on an individual fiber basis (C/F*i*) was determined as previously described (27). Images were taken at 20x with a CoolSNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY, USA). Images were analyzed

using Nikon NIS elements AR software. C/Fi was determined on an average of 50 fibres/animal.

Masson's Trichrome stain was used to determine collagen content in TA muscle cross sections. Sections were fixed in 4% PFA for 1 h then incubated in Bouin's fixative (Sigma, Oakville, ON, Canada) overnight at room temperature. Slides were rinsed in water, then incubated in Weigert's Iron hematoxylin for 5 min, washed again and incubated in biebrich scarlet - acid fuchsin for 15 min. Slides were then rinsed in water, incubated in phosphomolybdic-phosphotungstic acid 3x3min, incubated in aniline blue, dipped in water and incubated in 1% glacial acetic acid for 2 min. Slides were treated with graded ethanol washes then cover slipped. The area occupied by collagen (stained blue) was determined and represented as a percentage of total area. Images were taken at 20x using the Nikon DS-Fi1. Images were analyzed using Nikon NIS elements AR software

Oil-red-o stain was used to stain lipid in TA muscle cross sections. Slides were fixed for 1 h in 4% PFA, rinsed in water and incubated in oil red o working solution prepared with isopropanol for 30 min. Sections were rinsed in running water and cover slipped. Images were taken at 40x using the Nikon DS-Fi1. To quantify staining intensity was determined using using Nikon NIS elements AR software.

Statistical Analysis

To determine the impact of exercise training in old mice student's t-tests were run on all outcome measures between the O-Ex and the O-Sed groups at the CTL time point. To determine the impact of aging on muscle regeneration on all outcome measures a one way ANOVA was used to determine if there were differences between Y-Ctl and O-Sed at each time point (CTL, D10 and D28). To determine the impact of aging and exercise on all outcome measures a one way ANOVA was used to determine if there were differences between each group, Y-Ctl, O-Ex and O-Sed at each time point (CTL, D10, D28). Statistical significance was accepted at $P \leq 0.05$. Any significant main effects were analyzed using the Tukey's post-hoc test, alpha was adjusted based on the number of planned comparisons. All results were presented as means ± standard error of the mean (SEM).

Results.

1. Experimental Approach

The present study was designed to investigate whether exercise was able to improve muscle regeneration in old mice. Additionally, the experimental design allowed us to

determine the impact of exercise on old mice and the impact of age on muscle regeneration. Mice were 22 months old at the beginning of training (O-Ex). Mice trained 3 days/week for 8 weeks; mice were 24 months old at the end of the training protocol. A subset of mice (n=6) were sacrificed following training and their tibialis anterior (TA) muscles were isolated and frozen appropriately for further experiments. To determine the effect of exercise in old mice, TA muscles from O-Ex mice were compared to 24 month old sedentary mice that did not undergo any training (n=6). To determine the effect of aging on muscle regeneration, O-Sed animals were compared to 8 month old young mice (Y-Ctl). Mice were compared at baseline, prior to muscle injury (CTL), 10 d following injury (D10) and 28 d following injury (D28). To determine the effect of exercise training on muscle regeneration TA muscles isolated from Y-Ctl, O-Ex and O-Sed were compared at baseline, 10 and 28 days following injury.

2. The effect of exercise in old mice.

Fibre CSA was determined using immunofluorescent staining for laminin (FIG 1 A-I). Eight weeks of endurance exercise training did not lead to a change in fibre CSA of old mice (CTL: OEx: 2486 ± 125 μm2; O-Sed: 2275 ± 155 μm2) ($p>.05$) (FIG 1J). The extent of muscle regeneration was determined by the number of fibers containing centrally located nuclei (FIG 1 A-I), a hallmark of regenerating fibres (28). Old mice that underwent exercise training had a greater number of regenerating fibers (10 ± 2%) compared to old sedentary mice (4 ± 1%) ($p<.05$) (FIG 1 K). Although the exercise training protocol did not lead to an increase in CSA it did induce fibre regeneration.

SC content was determined with immunofluorescent staining of laminin, Pax7 and DAPI (FIG 1 A-I). Old mice that underwent exercise training had significantly more SC than old sedentary mice (CTL: O-Ex: 2.6 ± 0.4; O-Sed: 1.0 ± 0.1 SC/100 fibres) ($p<.05$) (FIG 1 L).

3. The effect of age on regeneration.

Regeneration is impaired in old mice

Fibre CSA was significantly reduced in both Y-Ctl and O-Sed mice 10 days following injury compared to CTL (CTL: Y-Ctl: 2475 ± 469, O-Sed: 2275 ± 155 μm2; D10: Y-Ctl: 1482 ± 167, O-Sed: 1086 ± 79 μm2) ($p<.05$). However, 28 days following injury CSA was re-established in Y-Ctl (D28: 2551 ± 226 μm2), whereas it remained reduced in O-Sed (1351±121 μm2) ($p<.05$) (FIG 1M). Additionally, the percentage of

regenerating fibres was significantly smaller in O-Sed (53 ±6%) 28 days following injury compared to Y-Ctl (76 ± 5%) (p=.05) (FIG 1 N).

The SC response to regeneration is impaired in old mice

SC content was greater 10 days following injury compared to CTL in both Y-Ctl (CTL: 2.6 9.1 ± 1.3SC/100 fibres) and O-Sed (CTL: 1.0 ±0.1; D10: 5.7 ±0.8 SC/100fibres) (p<.05). Twenty-eight days following injury SC content remained elevated in Y-Ctl compared to CTL (CTL: 2.6±0.4; D28: 6.7 ± 1.1 SC/100 fibres) (p<.05). No difference in SC content was observed in O-Sed between CTL (1.0 ±0.1 SC/100 fibres) and D28 (2.4 ± 0.5 SC/100 fibres) (p>.05) (FIG 1 O).

The revascularization process during regeneration is impaired in old mice

The number of capillaries per individual fibre (C/Fi) was determined via immunofluorescent staining of TA muscle cross section for laminin and CD31 (FIG 3 E-F). C/Fi was not different between Y-Ctl and O-Sed mice at CTL and 28 days following injury (p>.05) (FIG 3 I). VEGF mRNA expression was increased in both Y-Ctl and O-Sed groups 10 days following injury approximately 3- and 5-fold respectively group (p<.05) (FIG 3 J).

Fibrosis is increased in old mice during regeneration

In O-Sed the fibrotic index was greater 10 days (8.1 ±0.9%) following injury compared to CTL (3.4 ± 1%) (p<.05). The fibrotic index was greater in the O-Sed group (8.1 ± 0.9%) compared to the Y-Ctl (4.6 ±1%) group 10 days following injury (p<.05). No differences in fibrotic index were observed in the Y-Ctl group across any time point. Aging did not affect fibrosis as no differences existed at CTL between the Y-Ctl and O-Sed group at baseline (p>.05) (FIG 4K).

4. The effect of exercise on regeneration in old mice.

Exercise rescues impaired regeneration in old mice

C_S_A_ _w_a_s_ _r_e_d_u_c_e_d_ _i_n_ _a_l_l_ _g_r_o_u_p_s_ _1_0_ _d_a_y_s_ _f_o_l_l_o_w_i_n_g_ _i_n_j_u_r_y_ _c_o_m_p_a_r_e_d_ _t_o_ _C_T_L_ _(_p_<_._0_5_)_._ _C_S_A_ _w_a_s_ _r_e_-_e_s_t_a_b_l_i_s_h_e_d_ _t_o_ _C_T_L_ _v_a_l_u_e_s_ _2_8_ _d_a_y_s_ _f_o_l_l_o_w_i_n_g_ _i_n_j_u_r_y_ _i_n_ _Y_-_C_t_l_ _a_n_d_ _O_-_E_x_ _b_u_t_ _n_o_t_ _i_n_ _O_-_S_e_d_ _(_C_T_L_:_ _Y_-_C_t_l_:_ _2_4_7_5_ _±_ _2_6_5_ _µm_2_;_ _O_E_x_:_ _2_4_8_6_ _±_ _1_2_5_ _µm_2_;_ _O_-_S_e_d_:_ _2_2_7_5_ _±_ _1_5_5_ _µm_2_;_ _D_2_8_:_ _Y_:_ _2_5_5_1_ _±_ _2_2_6_ _µm_2_;_ _O_E_x_:_ _2_2_0_8_ _±_ _1_6_9_ _µm_2_;_ _O_-_S_e_d_:_ _1_3_5_1_ _±_ _1_2_1_

_µm_2_)_ _(_F_I_G_ _2_ _A_)_._ _T_h_e_ _p_e_r_c_e_n_t_a_g_e_ _o_f_ _r_e_g_e_n_e_r_a_t_i_n_g_ _f_i_b_e_r_s_ _w_a_s_ _g_r_e_a_t_e_r_ _i_n_ _a_l_l_ _g_r_o_u_p_s_ _1_0_ _d_a_y_s_ _f_o_l_l_o_w_i_n_g_ _i_n_j_u_r_y_ _(_p_<_._0_5_)_._ _A_d_d_i_t_i_o_n_a_l_l_y_,_ _t_h_e_ _p_e_r_c_e_n_t_a_g_e_ _o_f_ _r_e_g_e_n_e_r_a_t_i_n_g_ _f_i_b_r_e_s_ _w_a_s_ _s_m_a_l_l_e_r_ _a_t_ _D_2_8_ _i_n_ _t_h_e_ _O_-_S_e_d_ _g_r_o_u_p_ _(_5_3_ _±_ _6_%_)_ _c_o_m_p_a_r_e_d_ _t_o_ _t_h_e_ _Y_-_C_t_l_ _g_r_o_u_p_ _(_7_6_ _±_ _5_%_)_ _(_p_=_._0_5_)_ _(_F_I_G_ _2_ _B_)_._ _

The SC response during regeneration in old mice

SC content was greater 10 days following injury in all groups: Y-Ctl (CTL: 2.6 ± 0.4; D10: 9.1 ± 1.3 SC/100 fibres), O-Ex (CTL: 2.3 ± 0.3; D10: 7.9 ± 1.7 SC/100 fibres), and O-Sed (CTL: 1.0 ± 0.1; D10: 5.7 ± 0.5 SC/100 fibres) ($p<.05$) (FIG 2 C).

Exercise improves revascularization during regeneration in old mice

C/Fi was greater 28 days following injury in the O-Ex group only (CTL: 2.0 ± 0.1; D28: 2.6 ± 0.2 cap/individual fibre) ($p<.05$) (FIG 3 K). Additionally, C/Fi was greater in the O-Ex (2.6 ± 0.2 cap/individual fibre) 28 days following injury compared to both the O-Sed (1.8 ± 0.17 cap/individual fibre) and the Y-Ctl group 1.9 ± 0.1 cap/individual fibre) ($p<.05$) (FIG 3 K). VEGF mRNA expression was increased 10 days following injury in both the Y-Ctl and O-Ex group ($p<.05$) (FIG 3 L) and returned to CTL levels 28 days following injury.

Exercise does not impact lipid or collagen content during regeneration in old mice

Aging and/or exercise conditioning during regeneration did not significantly impact lipid content as assessed by oil red o staining in muscle cross section (FIG 5). Additionally aging and/or exercise during regeneration did not affect collagen expression as assessed via Mason's trichrome staining of muscle cross sections (FIG 4).

Discussion

For the first time, we demonstrate that exercise conditioning can rescue age-associated impaired muscle regeneration. Specifically, exercise-conditioned old mice re-established muscle fibre CSA 28 days following injury to the same extent as young animals. Consistent with previous reports, the exercise training protocol employed in this study resulted in an increase in SC content in old mice (FIG 1 L) without leading to an increase in muscle fibre CSA (FIG 1 J) (17,19). Although there

is no clear consensus on the ability of skeletal muscle from old mice to regenerate following injury, we observe incomplete regeneration in O-Sed animals 28 days following injury (FIG 2 A).

Exercise training results in remodelling of skeletal muscle tissue. These adaptations may, in part, explain the improvements observed in muscle regeneration in the O-Ex group. Here, we demonstrate that exercise resulted in an increase in SC content in old animals (FIG 1 L). SC are indispensible for skeletal muscle regeneration (4,5) and the increase in SC content in the O-Ex group as compared to that of the O-Sed group likely contributed to the similar regenerative pattern observed in both the O-Ex and Y-Ctl groups. The increased SC response in the Y-Ctl group persisted throughout the regeneration timeline, whereas this was not observed in the O-Sed group (FIG 1 O). Upon activation, SC proliferate; some differentiate and donate their nuclei to repair damaged fibers or fuse to one another to establish new fibers, while others revert to quiescence in order to maintain the SC pool (6,28). The greater SC content of young animals 28 days following injury that was not observed in the old sedentary group further demonstrates that skeletal muscle from old animals does not respond to injury in the same manner as young animals. The difference in SC content 28 days following injury likely contributed to the inability to completely restore CSA in old sedentary animals.

A hallmark of regenerating muscle is the presence of centrally located nuclei (28). A greater proportion of regenerating fibers were observed at CTL in O-Ex compared to O-Sed animals (FIG 1 K) suggesting that the exercise protocol was demanding enough to require some degree of skeletal muscle adaptation/repair. Furthermore, we reported incomplete regeneration, as assessed by fibre CSA, in O-Sed animals. However the proportion of regenerating fibers 28 days following injury was smaller in the O-Sed group compared to the Y-Ctl group (FIG 1 B) This difference was not observed between the O-Ex and the Y-Ctl group. Fewer regenerating fibres at D28 would suggest that the regenerative process was slowing in the O-Sed compared to the Y-Ctl group although fibre CSA was yet to be re-established to CTL group levels. Therefore, it is possible that the smaller fibre CSA observed in the O-Sed was not simply due to a delay in muscle regeneration but also that it may never be fully re-establish in light of the slowing indices of regeneration observed in this group. These findings are contradictory to previous work that reported a re-establishment of fibre CSA following injury in old mice (8). These authors induced injury via notexin, which

causes degeneration via a similar mechanism as CTX (29). However, the use of different protocols may explain the differences observed between studies as, unlike the current study, injury was induced in the extensor digitorum longus (EDL) of female C57BL/6J mice.

Revascularization is an important process during muscle regeneration (30), however, reports evaluating capillarization in old rodents are conflicting. A reduction (31,32), no change (31) and even an increase (31,33) in capillarization during muscle fibre regeneration have all been reported. These differences may, in part, be attributed to the difference in rodent model used (mouse versus rat) and muscle examined. Exercise is able to increase capillary content, albeit to a lesser extent, in older animals compared to young (34). Here, we report no difference in C/Fi between each group at baseline (FIG 3 K). However, we report a greater C/Fi in O-Ex compared to both the O-Sed and the Y-Ctl group 28 days following injury (FIG 3 K). Enhanced vascularization 28 days following injury in the O-Ex group presumably provides improved support during myogenesis, which was impaired in the O-Sed group. Improved perfusion of the skeletal muscle may allow for enhanced exposure to the circulating systemic environment resulting in improved support of muscle regeneration in exercise-conditioned animals. In contrast, we do not observe an increase in C/Fi in the Y-Ctl group following injury. It is possible that the last time-point (28 days) of our timeline was too late to observe an increase in the Y-Ctl group. Additionally, it is possible that the increase in C/Fi of the O-Ex group may be a compensatory mechanism. Muscle from old animals may require an even greater perfusion as compared to that of young animals to fully regenerate and we demonstrate in the current study that up-regulation of the angiogenic factor VEGF following injury is impaired in old sedentary animals (FIG 3 L). Our findings are supported by data that demonstrated impaired angiogenesis associated with reduced expression of VEGF following ischemic injury in old compared to young mice (32).

When animals are treated with a VEGF neutralizing antibody the associated increase in vascularisation following exercise is not observed (35), suggesting a role of VEGF in neovascularization. An increase in VEGF mRNA expression was observed 10 days following injury in the O-Ex and the Y-Ctl group, suggesting that muscle from these animals was expressing the appropriate cues to re-establish vascularisation

following injury. Due to the profound injury and incomplete muscle fiber formation, C/Fi was not analyzed 10 days following injury.

In addition to muscle fibre CSA we also aimed to determine the composition of skeletal muscle. Previous work has reported an increase in the fibrotic index in the early days following muscle injury in old mice (14). However, when regeneration is followed to later time points (i.e., 30 days following notexin injury) there were no observed differences in fibrotic index between young and old animals (8). Our results are in accordance with both of these studies. When determining the effect of aging on fibrosis during regeneration a greater fibrotic area was observed 10 days following injury in the O-Sed group compared to baseline (CTL) whereas the difference was no longer apparent 28 days following injury (FIG 4 K). However, when analyzing whether aging and exercise had an effect on fibrosis during regeneration, post-hoc tests revealed no differences between groups or time points. This may be due to lack of statistical power. We also determined lipid content as lipid infiltration of skeletal muscle increases with age in humans (36). Lee et al 2013 reported an increase in lipid deposits in old and senescent mice 30 and 21 days respectively following injury (8). Although not significant, lipid staining intensity was 80% greater in the O-Sed group 28 days following injury compared to baseline (CTL) whereas it was 14 and 37% greater in the Y-Ctl and O-Ex group's respectively (FIG 5 L). The content of non-muscle tissue (i.e. lipid and collagen) in skeletal muscle may impact regeneration and ultimately impair the re-establishment of fibre CSA and this seems to be especially true in O-Sed animals. Determining if the functional capacity of O-Ex mice was improved as compared to O-Sed mice would have been beneficial in determining if the re-establishment of fibre CSA translated to functional measures like strength and fatigability. In addition, an excess of non-contractile tissue such as adipose and connective tissue in skeletal muscle could also impact tissue contractility and result in reduced functional capacity.

Skeletal muscle has an outstanding ability to fully regenerate following traumatic injury. However, the ability of skeletal muscle to completely regenerate following injury has, on occasion, reported to be delayed or impaired in old animals (12–14). These findings are confirmed in the present study with O-Sed animals displaying impaired muscle fibre regeneration 28 days following injury. Here, however, we demonstrate that 8 weeks of progressive endurance training is able to rescue impaired muscle regeneration of old mice following CTX injury. Rescued

regeneration was likely a result of increased SC content following exercise. In addition, exercised animals appeared to have an improved re-establishment of the vascular network in response to exercise, which may have also contributed to complete skeletal muscle regeneration observed in this group. In conclusion, exercise-conditioning rescues delayed skeletal muscle regeneration observed in advanced age.

Acknowledgments

The Pax7 hybridoma cells developed by Dr. A. Kawakami were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA52242 USA.

Figure legend

Figure 1. Representative images of TA muscle cross sections from O-Ex (A-C), O-Sed (D-F) and Y-Ctl (G-I) at CTL (A,D,G), D10 (B,E,H) and D28 (C,F,I) stained for laminin (green), Pax7 (red) and nuclei (DAPI-blue). White circles indicate SC (Pax7 positive nuclei). The effect of exercise on CSA (J), the percentage of regenerating fibres (K) and on SC content (L) are presented at CTL in the O-Ex and O-Sed group. To determine the effect of age on regeneration CSA (M), the percentage of regenerating fibres (N) and SC content (O) are presented at CTL, D10 and D28 in the Y-Ctl and O-Sed groups. * significantly different from O-Ex, ** significantly different from Y-Ctl, *** significantly different that CTL within the same group.

Figure 2. The effect of exercise and aging on CSA, the dotted line represents average CSA at CTL of Y-Ctl, O-Ex and O-Sed, (A), the percentage of regenerating fibres (B) and on SC content (C) following regeneration are presented in Y-Ctl, O-Ex and O-Sed. Graphs of fibre CSA distribution at CTL (D), D10 (E) and D28 (F). Distribution shows a leftward shift of the curve in all groups at D10 indicating a greter number of smaller fibers, at D28 there is a rightward shift of the curve in the Y-Ctl and O-Ex group indicating re-establishment of CSA to CTL values. *** significantly different that CTL within the same group, b significantly different that O-Sed and D10 within the same group, ** significantly different from Y-Ctl.

Figure 3. Representative images of TA muscle cross sections from O-Ex (A,B), O-Sed (C,D) and Y-Ctl (E,F) stained for laminin (purple) and CD31 (green). The effect of exercise on C/Fi (G) and VEGF mRNA expression (H) are presented at CTL. The effect of aging and regeneration on C/Fi (I) and VEGF mRNA expression (J) are presented at CTL and D28 and CTL, D10 and D28 respectively. The effect of exercise and aging on C/Fi (K) and VEGF mRNA expression (L) during regeneration are presented in Y-Ctl, O-Ex and O-Sed. *** significantly different that CTL within the same group, c significantly different than D10.

Figure 4. Representative Masson trichrome images of TA muscle cross sections, from O-Ex (A-C), O-Sed (D-F) and Y-Ctl (G-I) at CTL (A,D,G), D10 (B,E,H) and D28 (C,F,I) from collagen is stained blue. The effect of exercise (J), aging and regeneration (K) and exercise and aging during regeneration (L) on collagen content are represented. *** significantly different that CTL within the same group, ** different than Y-Ctl.

Figure 5. Representative Oil-red-o images of TA muscle cross sections, from O-Ex (A- C), O-Sed (D-F) and Y-Ctl (G-I) at CTL (A,D,G), D10 (B,E,H) and D28 (C,F,I) lipid is stained red. The effect of exercise (J), aging and regeneration (K) and exercise and aging during regeneration (L) on collagen content are represented.

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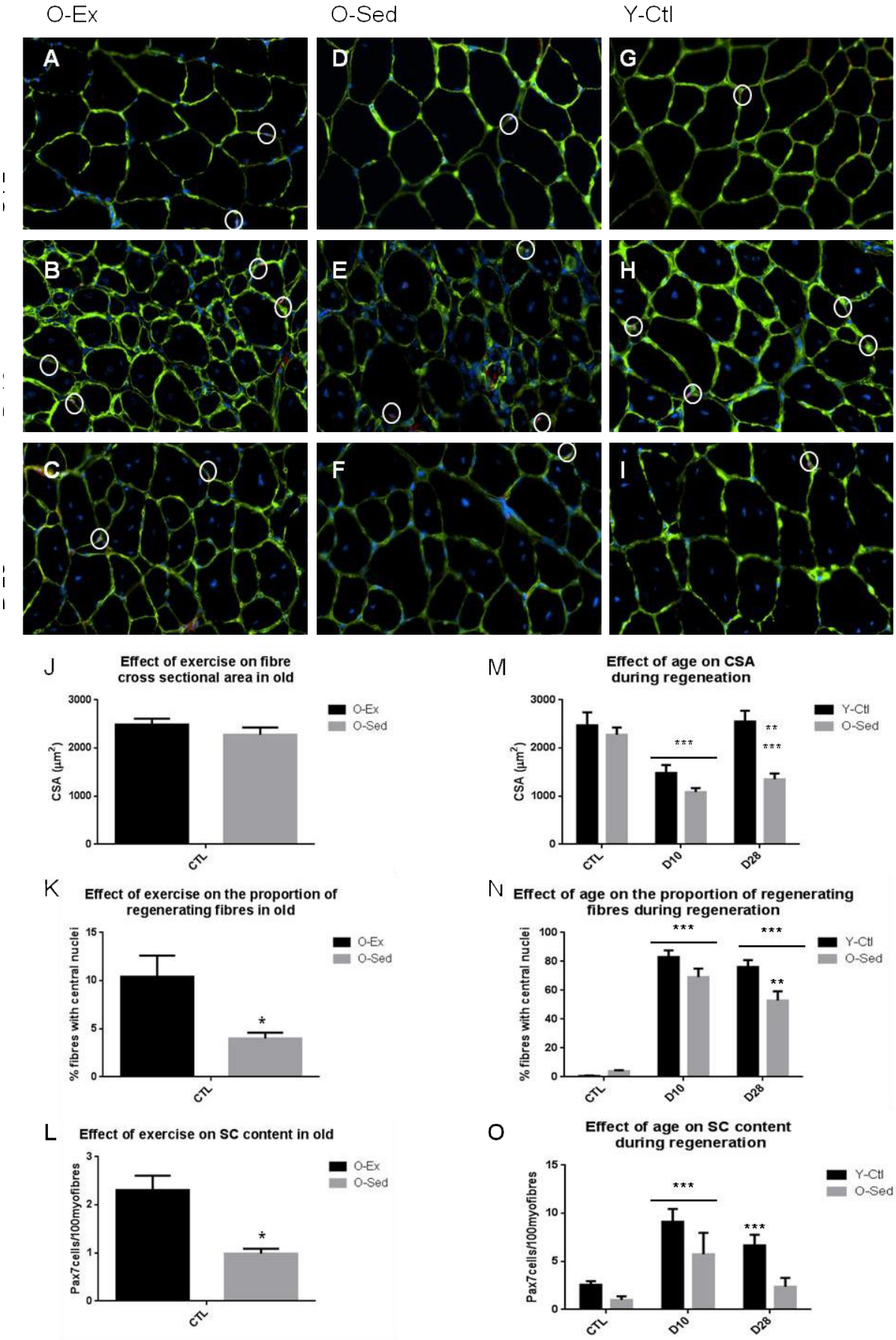
Figure 1


O-Ex
O-Sed
Y-Ctl
A
B
C
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H
I
J
Effect of exercise on fibre cross sectional area in old
CSA (µm²)
3000
2000
1000
0
CTL
O-Ex
O-Sed
M
Effect of age on CSA during regeneation
CSA (µm²)
CTL
D10
D28
***
**
***
Y-Ctl
O-Sed
K
Effect of exercise on the proportion of regenerating fibres in old
% fibres with central nuclei
15
10
5
0
*
CTL
O-Ex
O-Sed
N
Effect of age on the proportion of regenerating fibres during regeneration
% fibres with central nuclei
100
80
60
40
20
0
***
***
**
CTL
D10
D28
Y-Ctl
O-Sed
L
Effect of exercise on SC content in old
Pax7cells/100myofibres
3
2
1
0
*
CTL
O-Ex
O-Sed
O
Effect of age on SC content during regeneration
Pax7cells/100myofibres
15
10
5
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CTL
D10
D28
Y-Ctl
O-Sed

Figure 2

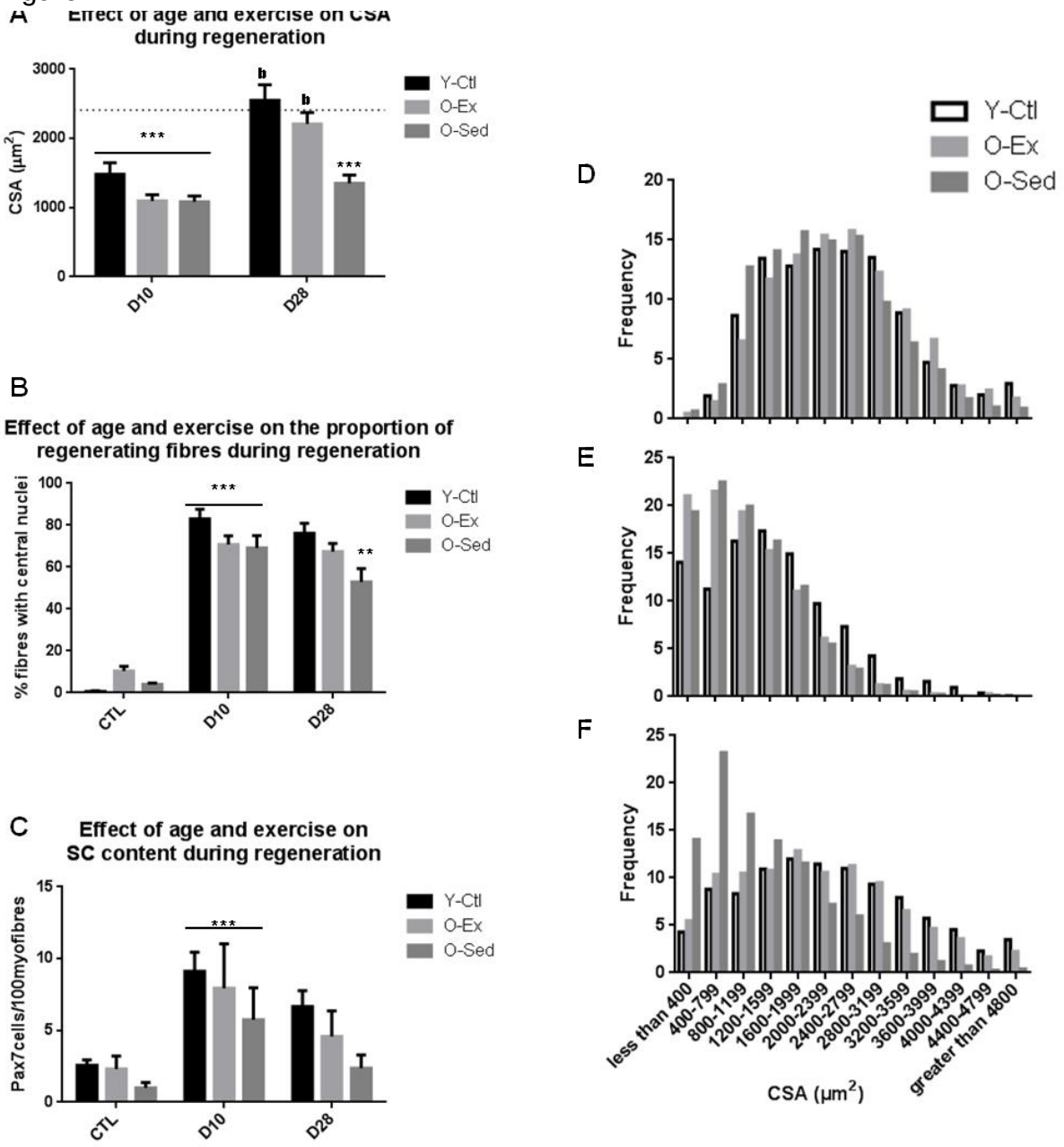
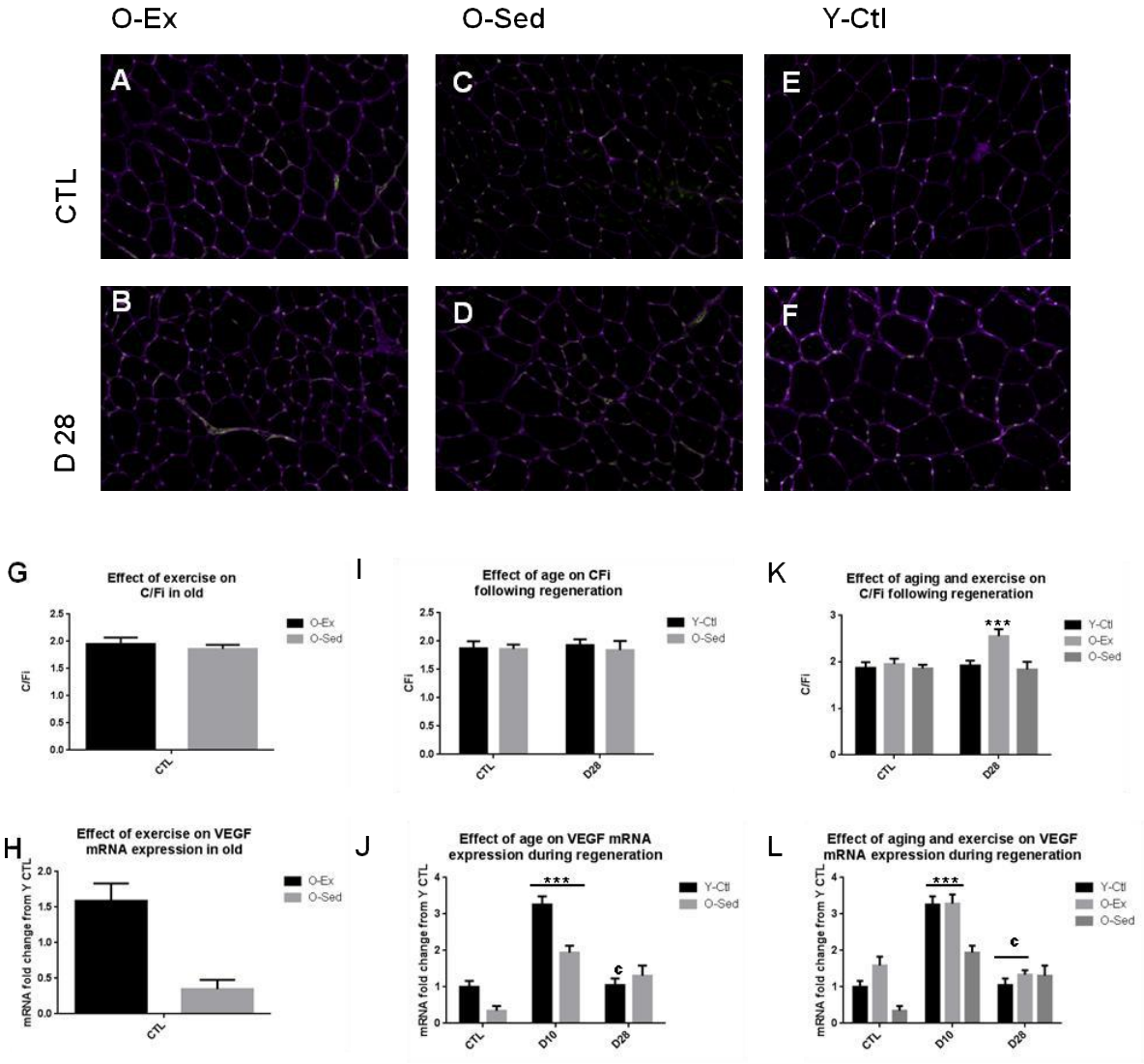

A
Effect of age and exercise on CSA during regeneration
CSA (µm²)
3000
2000
1000
0
***
b
b
***
D10
D28
Y-Ctl
O-Ex
O-Sed
B
Effect of age and exercise on the proportion of regenerating fibres during regeneration
% fibres with central nuclei
100
80
60
40
20
0
***
**
CTL
D10
D28
Y-Ctl
O-Ex
O-Sed
C
Effect of age and exercise on SC content during regeneration
Pax7cells/100myofibres
15
10
5
0
***
CTL
D10
D28
Y-Ctl
O-Ex
O-Sed
D
Frequency
20
15
10
5
0
E
Frequency
25
20
15
10
5
0
F
Frequency
25
20
15
10
5
0
less than 400
400-799
800-1199
1200-1599
1600-1999
2000-2399
2400-2799
2800-3199
3200-3599
3600-3999
4000-4399
4400-4799
greater than 4800
CSA (µm²)
Y-Ctl
O-Ex
O-Sed

Figure 3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O-Sed
Y-Ctl
A
C
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G
Effect of exercise on C/Fi in old
C/Fi
O-Ex
O-Sed
CTL
I
Effect of age on CFi following regeneration
CFi
Y-Ctl
O-Sed
CTL
D28
K
Effect of aging and exercise on C/Fi following regeneration
***
C/Fi
Y-Ctl
O-Ex
O-Sed
CTL
D28
H
Effect of exercise on VEGF mRNA expression in old
mRNA fold change from Y CTL
O-Ex
O-Sed
CTL
J
Effect of age on VEGF mRNA expression during regeneration
***
c
mRNA fold change from Y CTL
Y-Ctl
O-Sed
CTL
D10
D28
L
Effect of aging and exercise on VEGF mRNA expression during regeneration
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c
mRNA fold change from Y CTL
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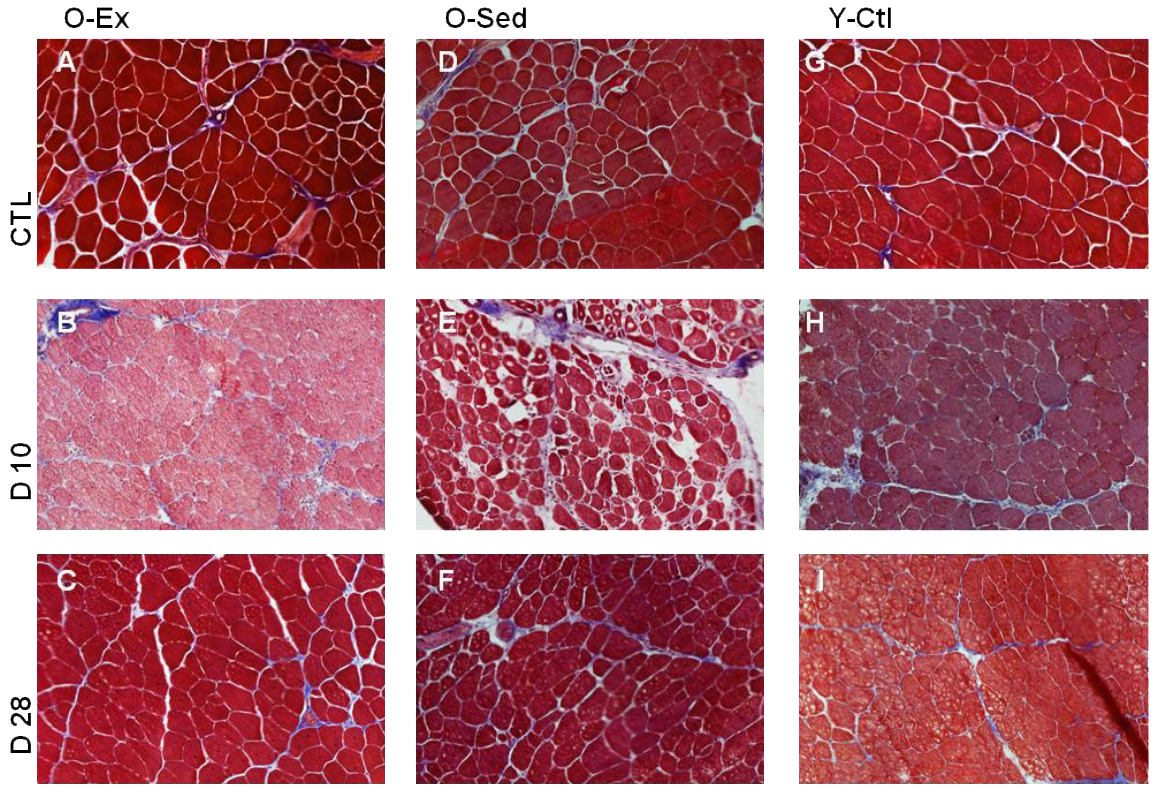
O-Ex
O-Sed
CTL
D10
D28

Figure 4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CTL
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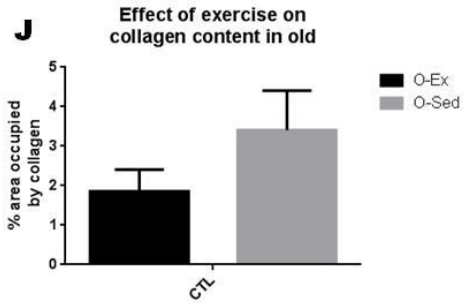
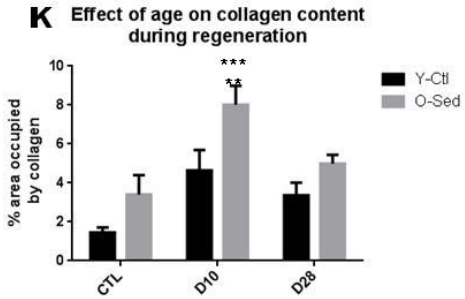

J
Effect of exercise on collagen content in old
% area occupied by collagen
O-Ex
O-Sed
CTL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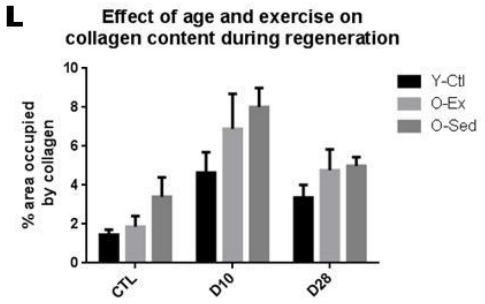
Effect of age on collagen content during regeneration
% area occupied by collagen
Y-Ctl
O-Sed
CTL
D10
D28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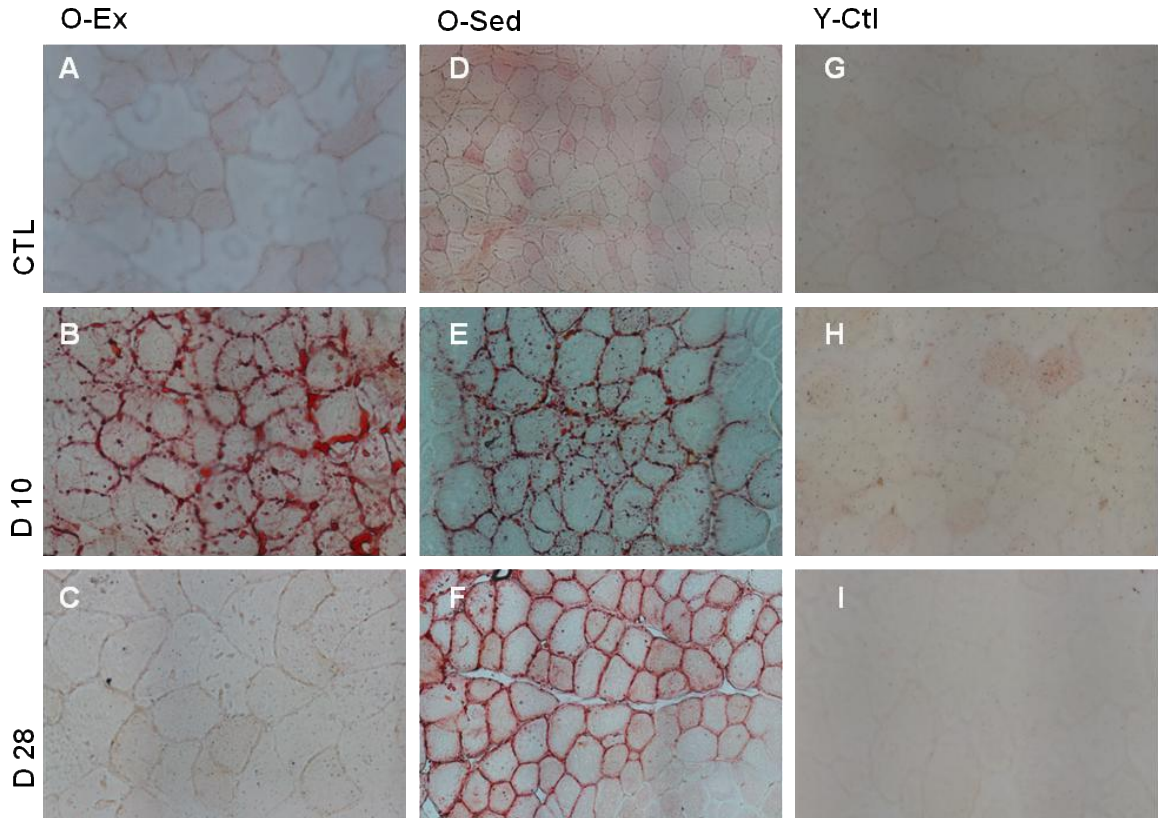
Effect of age and exercise on collagen content during regeneration
% area occupied by collagen
Y-Ctl
O-Ex
O-Sed
CTL
D10
D28

Figure 5


O-Ex
O-Sed
Y-Ctl
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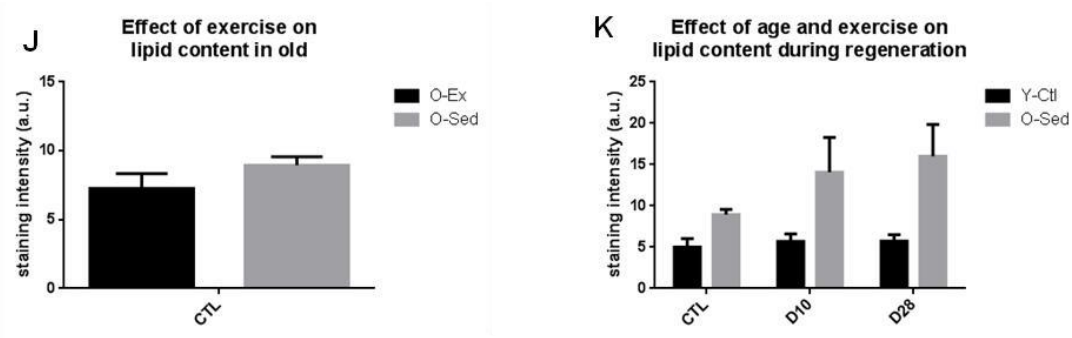
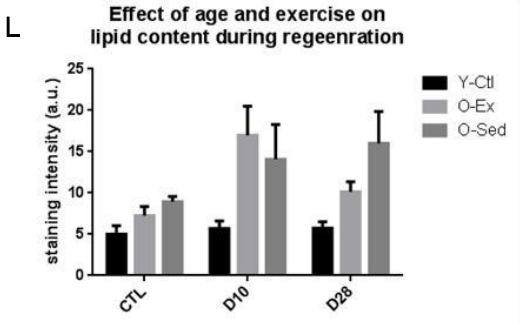

J
Effect of exercise on lipid content in old
staining intensity (a.u.)
O-Ex
O-Sed
CTL
K
Effect of age and exercise on lipid content during regeneration
staining intensity (a.u.)
Y-Ctl
O-Sed
CTL
D10
D28


L
Effect of age and exercise on lipid content during regeenration
staining intensity (a.u.)
Y-Ctl
O-Ex
O-Sed
CTL
D10
D28